

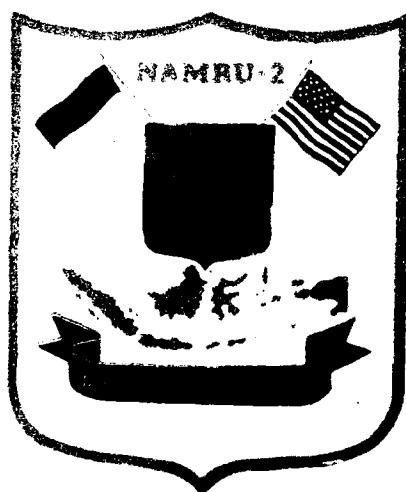
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FROM DIFFERENT REGIONS OF THE PHILIPPINES

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QUALITY CONTROL

IN VITRO GROWTH INHIBITION OF *PLASMODIUM FALCIPARUM* BY SERA FROM DIFFERENT REGIONS OF THE PHILIPPINES

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Abstract. Sera from different malaria endemic regions of the Republic of the Philippines were compared for their ability to inhibit growth of *Plasmodium falciparum* in vitro. Dialyzed serum was added to synchronous cultures containing schizonts for either the total 48 hr test period or only the last 24 hr in order to analyze the effects on erythrocytic invasion and intraerythrocytic growth, respectively. Reduction in ^3H -hypoxanthine uptake was used to determine the percent of inhibition compared to nonimmune serum. One hundred seventy sera from Mindanao and Palawan in the South, the centrally located island of Mindoro, and Luzon in the North, were tested against 4 *P. falciparum* strains from the Philippines and 1 from Africa. Indirect fluorescent antibody titers were not predictive of inhibition. Inhibition of merozoite invasion rather than intraerythrocytic parasite growth is suggested by this study. Generally, sera were more inhibitory to parasite strains from the same geographical area than to those from more remote areas.

Several in vitro studies have been done to determine the effect of immune sera on malaria parasites using the incorporation of a radiolabeled compound as an indicator of parasite development.¹⁻³ This growth inhibition assay has been suggested as a means to gain a better understanding of the acquired immune response to malarial infection and as a better assessment of clinical immunity against malaria than fluorescent antibody titers.^{4,5}

A comparison of immunity to malaria in Sudan and Indonesia reported the presence of a non-immunoglobulin crisis form factor (CFF) in sera from Sudan vs. an immunoglobulin component in sera from Indonesia.⁶

In the present study, we have attempted to examine growth inhibition of malaria parasites by Philippine sera. We selected 4 malaria endemic sites to represent different geographical regions of the Philippines. Our culture-adapted parasite strains came from 2 areas in the Philippines, 1 in the North and 1 in the South. The growth inhibition assay was used to determine whether significant differences exist between immune sera from these different malaria endemic areas in the ability to inhibit the geographically different parasites.

MATERIALS AND METHODS

Study population

Serum samples were obtained from volunteers in the course of seroepidemiologic surveys. Sets

of sera were collected from eastern and western Palawan. Parasite rates for these areas were 15-30% positive by Giemsa stained thick smear, depending on seasonal rainfall. Indirect fluorescent antibody (IFA) positivity rates for malaria were >80%. Two sets of sera were from the island of Luzon. These areas were Nueva Ecija and Montalban in Rizal Province (Fig. 1) with malaria point prevalence rates of 22% and 35%, respectively. Antibody positivity rates (IFA) were 80-90%. The sera were from Mindoro (15% point prevalence) and Marawi in Mindanao island. The prevalence rate was unavailable for Marawi, but the IFA antibody positivity rate was 37%.⁷ The IFA positivity rate for Mindoro was also >80%. Populations in these areas are usually exposed to both *Plasmodium falciparum* and *P. vivax*. Infection with *P. malariae* occurs rarely.

Serum preparation

Blood samples drawn by venipuncture using siliconized vacutainers (Becton-Dickinson) were allowed to clot and serum was separated by centrifugation. Sera were frozen in liquid nitrogen or dry ice and transported to laboratory facilities in Manila where they were kept at -20°C until used. Before assay, sera were pre-treated at 56°C for 30 min and dialyzed twice in PBS and once in RPMI 1640 using tubings with 12,000-14,000 M_w cut-offs (Spectrapor). Sera were then filter-

PHILIPPINES

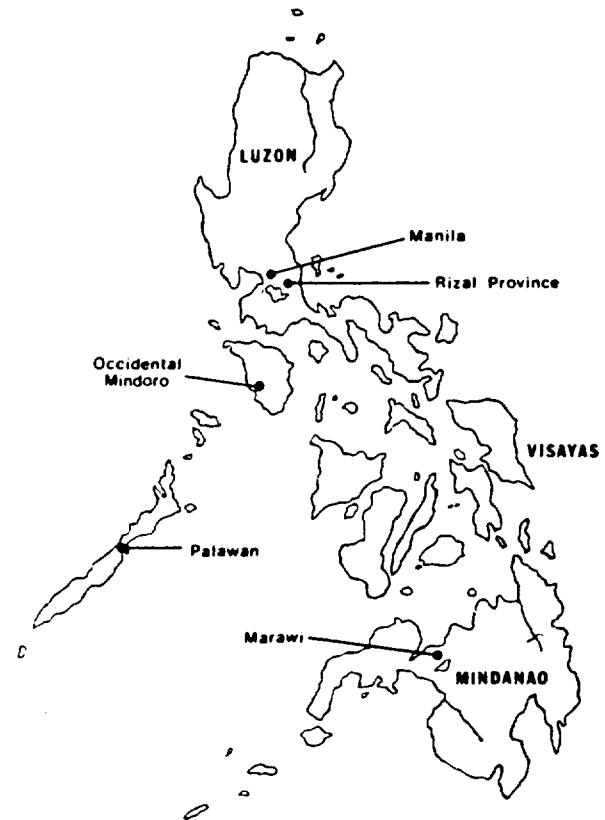


FIGURE 1. Map of the Republic of the Philippines showing serum collection sites.

sterilized and used at a final dilution of 25% in RPMI 1640.

Growth inhibition assay

The assay procedure followed was that of Jensen and others with minor modifications.⁸ Laboratory stock cultures of *P. falciparum* with >50 passages were used. A Palawan strain designated as 630, 3 strains from Rizal province (636, 644, and 888) and an African strain (G112) were used. Growth stages of the parasites were synchronized using a modified sorbitol lysis.⁹ Synchrony was done twice; the second wash with sorbitol was after a 12 hr incubation. Cultures were used at 1% parasitemia and 15% hematocrit at such time when the parasites became late segmenters. Growth medium was RPMI 1640 with 5% IFA malaria antibody negative human serum (RP5HS).

Each serum was assayed in 4 wells of a flat-bottomed 96-well microtiter plate: 2 for morphologic visualization and 2 for radiolabeling.

For each serum, 20 µl of parasite suspension was added to 1 morphologic and 1 radiolabeled well and grown for 24 hr in RP5HS medium containing 25% nonimmune serum (NS). The following 24 hr, the parasites were allowed to grow in RP5HS containing 25% immune serum (IS). The procedure is referred to as NS/IS. The change from NS to IS was done by aspirating the medium with a hypodermic needle when the cells had settled down after 24 hr incubation, and then adding the IS. The other wells likewise received 20 µl each of parasite suspension, but the suspension was grown for 48 hr in RP5HS containing 25% immune serum (IS/IS procedure). Fresh IS serum was added after the first 24 hr incubation. For the purpose of radiolabeling, 20 µl of RPMI containing 10 µCi/ml ³H-hypoxanthine was added after the first 24 hr. Unlabeled hypoxanthine was used for the morphologic wells. After 48 hr, all wells with ³H-hypoxanthine were harvested onto glass fiber filters and counted in a scintillation counter. Morphologic wells were used to prepare Giemsa-stained thin smears to determine parasite stages and their comparative counts.

In the assay proper, immune sera were processed simultaneously with at least 3 control sera. These control sera were obtained from volunteer laboratory personnel with no known history of residing in malaria endemic areas and who tested negative for malaria antibody on the indirect fluorescent antibody test. Finally, percent inhibition was calculated for the radiolabeled wells by comparing radioactivity counts obtained from the wells containing the immune sera with average counts obtained from wells containing control sera.

Indirect fluorescent antibody test

All serum samples were tested for IgG antibodies against malaria using a standard IFA technique.¹⁰ The parasite used for the IFA test was a *P. falciparum* strain obtained from Palawan and grown in continuous culture in the laboratory.

RESULTS

Percent inhibition data are shown in Table 1. Most of the inhibition was demonstrated in the IS/IS wells, except in the cases of Mindoro sera against Luzon strains of parasites and, to a lesser extent, Luzon sera against all strains of parasites.

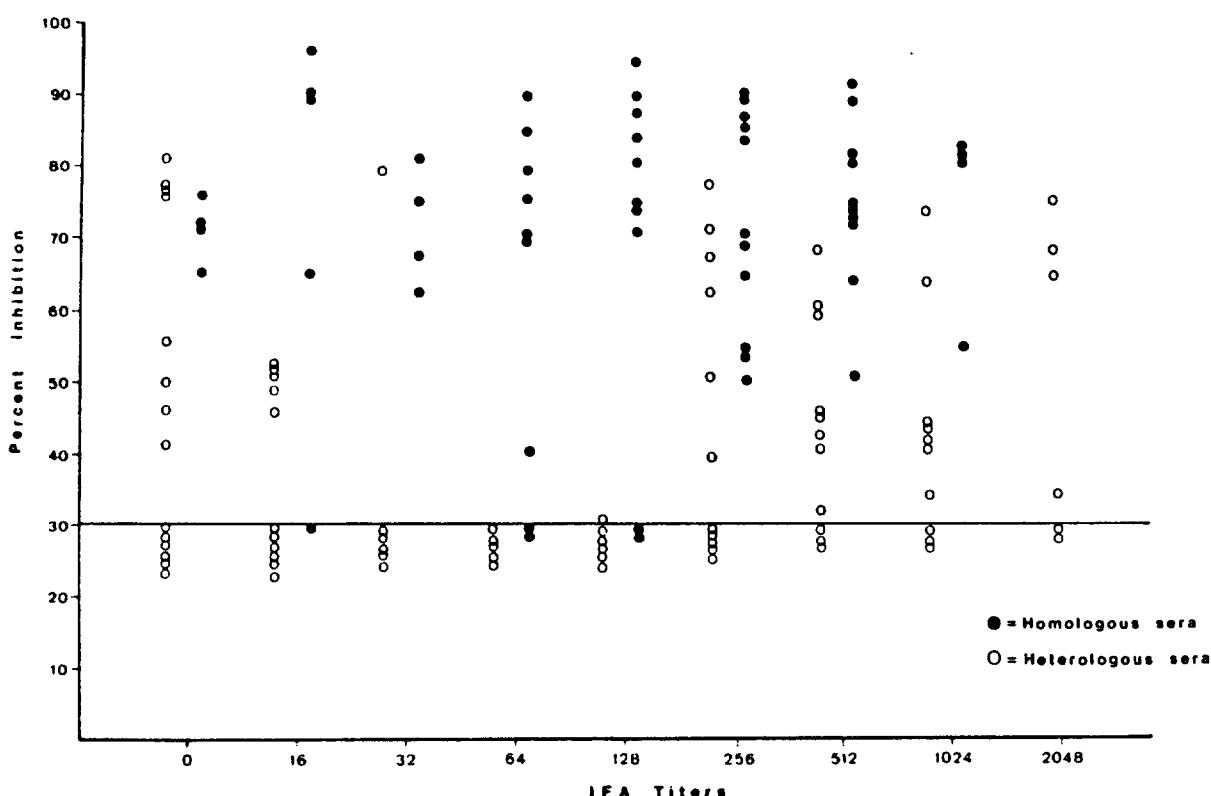


FIGURE 2. Comparison of titers and inhibitory effects of sera from selected malaria endemic areas. Parasite strain used: 630 (Palawan strain), 48 hr incubation.

Homologous sera, defined as sera taken from the same area as the parasite, showed significantly better inhibition of parasite growth than heterologous sera in the IS/IS wells. Significantly greater number of sera from Palawan were able to inhibit parasites ($\geq 30\%$ inhibition) from Palawan than Luzon or Africa ($\chi^2, P < 0.01$). This was also noted for Luzon sera vs. Luzon strains of parasites as compared to Palawan or African strains ($\chi^2, P < 0.01$).

Indirect fluorescent antibody titers did not correlate with percent inhibition shown by the test sera. Sera with titers in the range of 1:128 and above were not necessarily predictive of better inhibition of parasite growth, and sera with lower titers did not necessarily exhibit less inhibition (Fig. 2).

Figure 3 represents responses of different sera tested against the different parasite strains used. Percent inhibitions of these sera were figures obtained from the IS/IS wells. In general, good inhibitions were demonstrated from the homologous sera as compared to the heterologous sera. Also, there were fewer numbers of homologous sera which did not favorably inhibit the parasite strain ($< 30\%$ inhibition).

DISCUSSION

One hundred seventy-four sera from different regions in the Philippines endemic for malaria showed a wide variability with regard to their ability to inhibit growth of *P. falciparum* in vitro. However, there was a consistent pattern in that most of the inhibition was demonstrated in the IS/IS wells. This suggests that inhibition was primarily due to interference with merozoite-erythrocytic interaction rather than effects on intra-erythrocytic parasite development. Examination of the parasites in the IS/IS morphologic wells supported this observation, since there was a reduction in the number of surviving parasites exposed to immune sera when compared to the control wells.

Sera from most areas exhibited greater inhibition of homologous strains than of heterologous strains. These data suggest that, although geographically quite close, the parasites may show immunological differences. Migration among even semi-immune inhabitants could result in a significantly increased risk of acquiring malaria and an individual with strong immunity to local isolates of *P. falciparum* may have little or no protection against those from other countries or

TABLE I
In vitro growth inhibition of P. falciparum by sera from different regions in the Philippines

Strains of <i>P. falciparum</i>	Test sera added	Palawan sera				Mindoro sera				Luzon sera				Marawi sera												
		No. *	Max†	Mean ± SD‡	No. *	Max†	Mean ± SD‡	No. *	Max†	Mean ± SD‡	No. *	Max†	Mean ± SD‡	No. *	Max†	Mean ± SD‡										
(Palawan)	NS/IS§	24/61	79.2	47.4 ± 14.2	21/51	82.1	48.6 ± 15.9	16/41	88.3	49.4 ± 17.2	7/20	53.8	40.8 ± 7.7	G1/12	NS/IS	2/31	76.4 ± 12.5	39/51	82.4 ± 14.8	12/41	82.3 ± 17.8	13/20	82.3 ± 14.6			
	IS/IS††	53/61**	97.2	76.4 ± 12.5	39/51	82.4	54 ± 14.8	12/41	82.3	58.7 ± 17.8	14/19	50.1	40.8 ± 11.6													
(Gambia)	NS/IS	2/31	40	37.5 ± 3.5	15/20	91.3	59.3 ± 16.8	10/20	79.6	47.3 ± 15.2	8/19	56.8	43.4 ± 9.6	636	NS/IS	20/31	55.7 ± 12.8	14/20	90.1	61.4 ± 17.5	7/20	89.2	45.3 ± 20.6	14/19	54.3 ± 11.6	
	IS/IS	76/2	55.7	55.7 ± 12.8	14/20	90.1	61.4 ± 17.5	7/20	89.2	45.3 ± 20.6	14/19	70	54.3 ± 11.6													
644	NS/IS	31/62	75.2	49.9 ± 12.5	14/31	85.3	60.9 ± 19.8	18/22	63.4	45.8 ± 10.4	ND (QNS)††	ND (QNS)	ND (QNS)	888	IS/IS	42/62	81.4	62.1 ± 11.9	8/31	61.3	41.6 ± 9.7	17/22**	96.2	64.5 ± 20.8	ND (QNS)	ND (QNS)
	IS/IS	42/62	81.4	62.1 ± 11.9	8/31	61.3	41.6 ± 9.7	17/22**	96.2	64.5 ± 20.8	ND (QNS)	ND (QNS)	ND (QNS)													
(Rizal, Luzon)																										

* No. of sera with ≥ 30% inhibition.

† Maximum percent inhibition.

‡ Mean ± SD inhibition.

§ NS/IS: test sera added only at 24 hr.

†† IS/IS: test sera added at 0 and 24 hr.

•• $\chi^2 = P < 0.01$ Palawan sera + Palawan strain vs. Palawan sera + Gambia and Luzon strain. $\chi^2 = P < 0.01$ Luzon sera + Luzon strain vs. Luzon sera + Gambia and Palawan strain.

†† Not done. Quantity not sufficient.

even another region in the same country. A study done in Madagascar seems to support this possibility, as bloods from 3 different endemic areas were shown to have diversified immunoprecipitation patterns when reacted against a particular falciparum strain.¹¹

Philippine sera generally had slight to moderate inhibition against African strains of parasites. Sera from Mindoro were slightly more inhibitory to African strains than other groups of sera tested. However, none of the Philippine sera was statistically different with respect to inhibition of this particular strain of parasite. Furthermore, increased inhibition was again demonstrated to occur in the IS/IS wells.

These observations are similar to findings in Indonesia¹² and Papua New Guinea.¹³ While growth inhibition in our study does not suggest major involvement of a CFF,¹⁴ this could not be entirely ruled out, particularly with respect to the Mindoro and Luzon sera. A number of these sera were found to inhibit NS/IS wells more than their IS/IS counterparts. A correlation, however, could not be specifically defined in as much as the presence of so-called crisis forms or deformed parasites was not consistently found. Morphological examinations of the NS/IS stained slides did show what might be described as deformed parasites. However, these deformed parasites were evident both in those with high NS/IS inhibition as well as in those with low NS/IS inhibition, suggesting that this might not be a crisis form activity. Furthermore, IFA titers of these same sera did not show a valid correlation. Crisis form activity, as shown in the morphological wells, was not evident as would be expected in those sera with low IFA titers. The general trend was that percent inhibition in the NS/IS wells dropped when the titers were also low. This was the general picture, but exceptions did occur in all of the sera tested.

Parasite variability could account for the diversity of immune response to malaria infection as evidenced by the wide divergence of the ability of immune sera to inhibit geographically distinct parasites.

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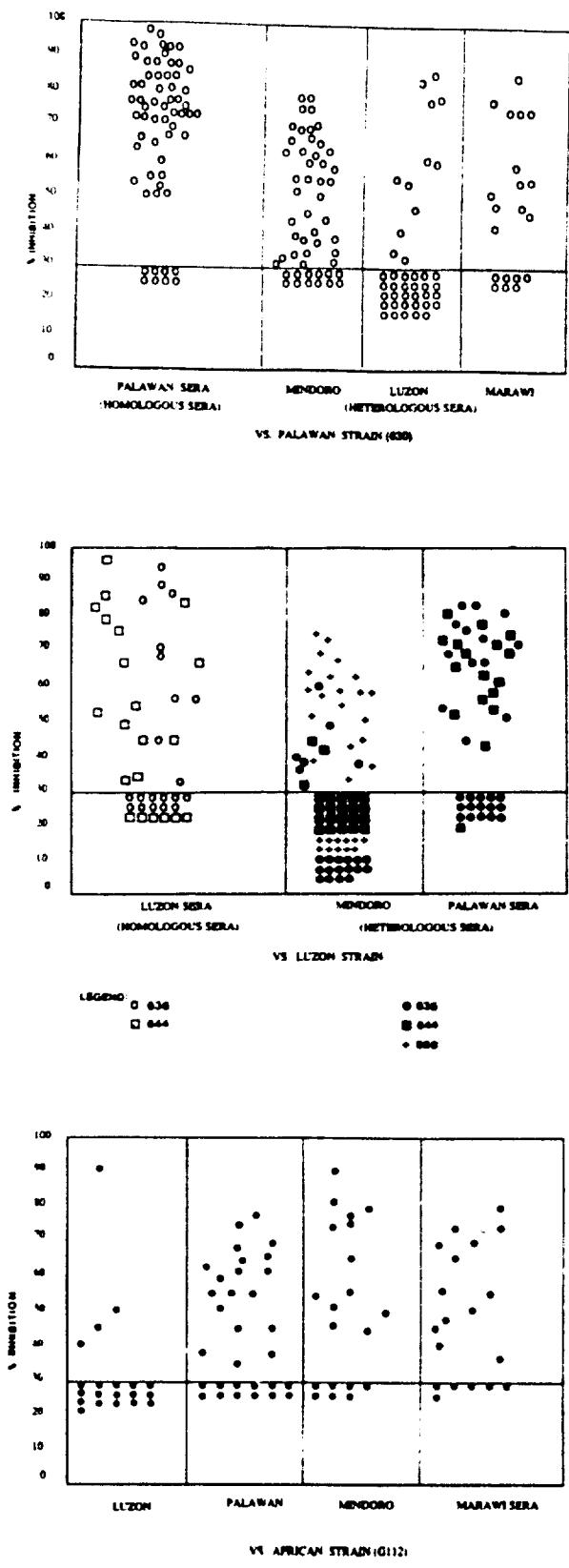


FIGURE 3. Distribution of growth inhibition activity of different sera against various strains of *P. falciparum* parasites (IS/IS wells).

REFERENCES

- Cohen S, Butcher GA, Crandall RB, 1969. Action of malarial antibody in vitro. *Nature* 223: 368-371. file m66; (ui) 70002154
- Cowen N, Clancy R, Cripps A, Alpers M, 1983. A microassay for detecting merozoite inhibition suitable for routine laboratory use. *Am J Trop Med Hyg* 32: 6-10. file m83; (ui) 83124440
- Stanley HA, Reese RT, 1984. In vitro inhibition of intracellular growth of *Plasmodium falciparum* by immune sera. *Am J Trop Med Hyg* 33: 12-16. file m83; (ui) 84125828
- Jensen JB, Boland MT, Allan JS, Carlin JM, Vande Waa JA, Divo AA, Akood MA, 1983. Association between human serum-induced crisis forms in cultured *Plasmodium falciparum* and clinical immunity to malaria in Sudan. *Infect Immun* 41: 1302-1311. file m83; (ui) 83289609
- Golenser J, Miller J, Avraham H, Spira DT, 1983. The inhibitory effect of human immune sera upon the in vitro development of *Plasmodium falciparum*. *Trop Geogr Med* 35: 15-20. file m83; (ui) 83303346
- Jensen JB, Hoffman SL, Boland MT, Akood MA, Laughlin LW, Kurniawan L, Marwoto HA, 1984. Comparison of immunity to malaria in Sudan and Indonesia: crisis-form versus merozoite-invasion inhibition. *Proc Natl Acad Sci U S A* 81: 922-925. file m83; (ui) 84144816
- Cross JH, Basaca-Sevilla V, 1984. *Biomedical surveys in the Philippines*. NAMRU-2 Special Publication no. 47, Manila, Philippines.
- Jensen JB, Boland MT, Hayes M, Akood MA, 1982. *Plasmodium falciparum*: rapid assay for in vitro inhibition due to human serum from residents of malarious areas. *Exp Parasitol* 54: 416-424. file m80; (ui) 83105543
- Lambros C, Vanderberg JP, 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* 65: 418-420. file m77; (ui) 80008554
- World Health Organization, 1974. Serological testing in malaria. *Bull World Health Organ* 50: 527-535. file m72; (ui) 75148519
- Deloron P, Jaureguiberry G, Le Bras J, Coulanges P, 1985. Geographic diversity of *Plasmodium falciparum* antibodies in Madagascar. *Lancet* 1: 444-447. file m83; (ui) 85136507
- Campbell JR, Hoffman SL, Leksana B, Kurniawan L, Marwoto HA, 1986. In vitro growth inhibition of *Plasmodium falciparum* by sera from tropical splenomegaly syndrome patients. *Am J Trop Med Hyg* 35: 708-710. file m86; (ui) 86266200
- Butcher GA, Clark IA, Crane G, 1987. Inhibition of intra-erythrocytic growth of *Plasmodium falciparum* by human sera from Papua New Guinea. *Trans R Soc Trop Med Hyg* 81: 568-572. file m86; (ui) 88178963
- Jensen JB, Boland MT, Akood M, 1982. Induction of crisis forms in cultured *Plasmodium falciparum* with human immune serum from Sudan. *Science* 216: 1230-1233. file m80; (ui) 82199449

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